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(71) Applicants *(for all designated States except US)*: **SMITHKLINE BEECHAM BIOLOGICALS S.A.** [BE/BE]; Rue de l'Institut 89, B-1330 Rixensart (BE). **CORIXA CORPORATION** [US/US]; 1124 Columbia Street, Suite 464, Seattle, WA 98104 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CABEZON SILVA, Teresa** [BE/BE]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B-1330 Rixensart (BE). **DILLON, Davin, Clifford** [US/US]; Corixa Corporation, 1124 Columbia Street, Suite 464, Seattle, WA 98104 (US).

(74) Agent: **DALTON, Marcus, Jonathan, William**; SmithKline Beecham, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).

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WO 01/04143 A3

(54) Title: PROSTASE VACCINE

(57) Abstract: The present invention relates to novel proteins and to their production, from the prostase family. In particular, the invention pertains to a prostase protein or fragment thereof fused to an immunological fusion partner, such as NS1. Such antigens may be formulated to provide vaccines for the treatment of prostate tumours. Novel methods for purifying prostase protein and homologues are also provided.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/62 C07K19/00 C12N1/21 A61K39/00 A61K31/70
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 37093 A (CORIXA CORP) 27 August 1998 (1998-08-27) claims ---	1-18
Y	NELSON PETER S ET AL: "Molecular cloning and characterization of prostase, an androgen-regulated serine protease with prostate-restricted expression." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 6, 16 March 1999 (1999-03-16), pages 3114-3119, XP002165774 March 16, 1999 ISSN: 0027-8424 the whole document --- -/--	1-18

[X] Further documents are listed in the continuation of box C.

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European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1997 HAUPL T ET AL: "Activation of monocytes by three OspA vaccine candidates: Lipoprotein OspA is a potent stimulator of monokines." Database accession no. PREV199799800815 XP002165775 abstract & FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY, vol. 19, no. 1, 1997, pages 15-23, ISSN: 0928-8244	1-18
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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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WO 01/04143 A2

(54) Title: VACCINE

(57) Abstract: The present invention relates to novel proteins and to their production, from the prostase family. In particular, the invention pertains to a prostase protein or fragment thereof fused to an immunological fusion partner, such as NS1. Such antigens may be formulated to provide vaccines for the treatment of prostate tumours. Novel methods for purifying prostase protein and homologues are also provided.

VACCINE

The present invention relates to protein derivatives of a protein known as prostase, a prostate-specific serine protease, to methods for their purification and manufacture, and also to pharmaceutical compositions containing such derivatives, and to their use in medicine. In particular such derivatives find utility in cancer vaccine therapy, particularly prostate cancer vaccine therapy and diagnostic agents for prostate tumours.

In particular the derivatives of the invention include fusion proteins comprising prostase linked to an immunological or an expression enhancer fusion partner.

The present invention also provides methods for purifying the prostase derivatives and for formulating vaccines for immunotherapeutically treating prostate cancer patients and prostase-expressing tumours other than prostate tumours, prostatic hyperplasia, and prostate intraepithelilial neoplasia (PIN).

Prostate cancer is the most common cancer among males, with an estimated incidence of 30% in men over the age of 50. Overwhelming clinical evidence shows that human prostate cancer has the propency to metastasise to bone, and the disease appears to progress inevitably from androgen dependent to androgen refractory status, leading to increased patient mortality (Abbas F., Scardino P. "The Natural History of Clinical Prostate Carcinoma." *In* Cancer (1997); 80:827-833). This prevalent disease is currently the second leading cause of cancer death among men in the US.

Despite considerable research into therapies for the disease, prostate cancer remains difficult to treat. Currently, treatment is based on surgery and/or radiation therapy, but these methods are ineffective in a significant percentage of cases (Frydenberg M., Stricker P., Kaye K. "Prostate Cancer Diagnosis and Management "The Lancet (1997); 349:1681-1687). Several tumour-associated antigens are already known. Many of these antigens may be interesting targets for immunotherapy, but are either not fully tumour-specific or are closely related to normal proteins, and hence bear with them the risk of organ-specific auto-immunity, once targeted by a potent immune response. When an auto-immune response to non-crucial organs can be tolerated, auto-immunity to heart, intestine and other crucial organs could lead to unacceptable safety profiles. Some previously identified prostate specific proteins like

prostate specific antigen (PSA) and prostatic acid phosphatase (PAP), prostate-specific membrane antigen (PSMA) and prostate stem cell antigen (PSCA) have limited therapeutic potential and moreover are not always correlated with the presence of prostate cancer or with the level of metastasis (Pound C., Partin A., Eisenberg M. et al. "Natural History of Progression after PSA Elevation following Radical Prostatectomy." *In* Jama (1999); 281:1591-1597) (Bostwick D., Pacelli A., Blute M. et al. "Prostate Specific Membrane Antigen Expression in Prostatic Intraepithelial Neoplasia and Adenocarcinoma." *In* Cancer (1998); 82:2256-2261).

The existence of tumour rejection mechanisms has been recognised since several decades. Tumour antigens, though encoded by the genome of the organism and thus theoretically not recognized by the immune system through the immune tolerance phenomenon, can occasionally induce immune responses detectable in cancer patients. This is evidenced by antibodies or T cell responses to antigens expressed by the tumour (Xue BH., Zhang Y., Sosman J. et al. "Induction of Human Cytotoxic T-Lymphocytes Specific for Prostate-Specific Antigen." *In* Prostate (1997); 30(2):73-78). When relatively weak anti-tumour effects can be observed through the administration of antibodies recognizing cell surface markers of tumour cells, induction of strong T cell responses to antigens expressed by tumour cells can lead to complete regression of established tumours in animal models (mainly murine).

It is now recognised that the expression of tumour antigens by a cell is not sufficient for *induction* of an immune response to these antigens. Initiation of a tumour rejection response requires a series of immune amplification phenomena dependent on the intervention of antigen presenting cells, responsible for delivery of a series of activation signals.

Prostase is a prostate-specific serine protease (trypsin-like), 254 amino acid-long, with a conserved serine protease catalytic triad H-D-S and a amino-terminal pre-propeptide sequence, indicating a potential secretory function (P. Nelson, Lu Gan, C. Ferguson, P. Moss, R. Gelinas, L. Hood & K. Wand, "Molecular cloning and characterisation of prostase, an androgen-regulated serine protease with prostate restricted expression, *In* Proc. Natl. Acad. Sci. USA (1999) 96, 3114-3119). A putative glycosylation site has been described. The predicted structure is very similar to other known serine proteases, showing that the mature polypeptide folds into a

single domain. The mature protein is 224 amino acids-long, with one A2 epitope shown to be naturally processed.

Prostase nucleotide sequence and deduced polypeptide sequence and homologs are disclosed in Ferguson, et al. (Proc. Natl. Acad. Sci. USA 1999, 96, 3114-3119) and in International Patent Applications No. WO 98/12302 (and also the corresponding granted patent US 5,955,306), WO 98/20117 (and also the corresponding granted patents US 5,840,871 and US 5,786,148) (prostate-specific kallikrein) and WO 00/04149 (P703P).

The present invention provides prostase protein fusions based on prostase protein and fragments and homologues thereof ("derivatives"). Such derivatives are suitable for use in therapeutic vaccine formulations which are suitable for the treatment of a prostate tumours.

Prostase fragments of the invention will be of at least about 10 consecutive amino acids, preferably about 20, more preferably about 50, more preferably about 100, more preferably about 150 contiguous amino acids selected from the amino acid sequences as shown in SEQ ID N°7 or SEQ ID N°8. More particularly fragments will retain some functional property, preferably a immunological activity, of the larger molecule set forth in SEQ ID N°7 or SEQ ID N°8, and are useful in the methods described herein (e.g. in vaccine compositions, in diagnostics, etc.). In particular the fragments will be able to generate an immune response, when suitable attached to a carrier, that will recognise the protein of SEQ ID N°7 or SEQ ID N°8.

Prostase homologues will generally share substantial sequence similarity, and include isolated polypeptides comprising an amino acid sequence which has at least 70% identity, preferably at least 80% identity, more preferably at least 90% identity, yet more preferably at least 95% identity, most preferably at least 97-99% identity, to that of SEQ ID NO:7 or SEQ ID N°8 over the entire length of SEQ ID NO:7 or SEQ ID N°8. Such polypeptides include those comprising the amino acid of SEQ ID NO:7 or SEQ ID N°8.

The prostase antigen derivative or fragments and homologues thereof may in a preferred embodiment carry a mutation in the active site of the protein, to reduce substantially or preferably eliminate its protease biological activity. Preferred mutations involve replacing the Histidine and Aspartate catalytic residues of the serine protease. In a preferred embodiment, prostase contains a Histidine-Alanine

mutation at residue 71 of prostase sequence (Ferguson, et al. (Proc. Natl. Acad. Sci. USA 1999, 96, 3114-3119) which corresponds to amino acid 43 of P703PDE5 sequence (SEQ ID N°8). The mutated protein preferably has a significant decrease in the catalytic efficiency (expressed in enzymatic specific activity) of the protein as compared to the non-mutated one. Preferably the reduction in the catalytic efficiency is at least by a factor of 10^3, more preferably at least by a factor of 10^6. The protein which has undergone a histidine alanine mutation is hereafter referred to as * (star).

In one embodiment, the present invention relates to fusions proteins, comprising the tumour-associated prostase or fragment or homologues thereof and a heterologous protein or part of a protein acting as a fusion partner. The protein and the fusion partner may be chemically conjugated, but are preferably expressed as recombinant fusion proteins in a heterologous expression system.

In a preferred embodiment of the invention there is provided a prostase fusion protein or fragment or homologues thereof linked to an immunological fusion partner that may assist in providing T helper epitopes. Thus the fusion partner may act through a bystander helper effect linked to secretion of activation signals by a large number of T cells specific to the foreign protein or peptide, thereby enhancing the induction of immunity to the prostase component as compared to the non-fused protein. Preferably the heterologous partner is selected to be recognizable by T cells in a majority of humans.

In another embodiment, the invention provides a prostase protein or fragment or homologues thereof linked to a fusion partner that acts as an expression enhancer. Thus the fusion partner may assist in aiding in the expression of prostase in a heterologous system, allowing increased levels to be produced in an expression system as compared to the native recombinant protein.

Preferably the fusion partner will be both an immunological fusion partner and an expression enhancer partner. Accordingly, the present invention in the embodiment provides fusion proteins comprising the tumour-specific prostase or a fragment thereof linked to a fusion partner. Preferably the fusion partner is acting both as an immunological fusion partner and as an expression enhancer partner. Accordingly, in a preferred form of the invention, the fusion partner is the non-structural protein from influenzae virus, NS1 (hemagglutinin) or fragment thereof. Typically the N-terminal 81 amino acids are utilised, although different fragments

may be used provided they include T-helper epitopes (C. Hackett, D. Horowitz, M. Wysocka & S. Dillon, 1992, J. Gen. Virology, 73, 1339-1343). When NS1 is the immunological fusion partner it has the additional advantage in that it allows higher expression yields to be achieved. In particular, such fusions are expressed at higher yields than the native recombinant prostase proteins.

In preferred embodiments, the prostase môiety within the fusion is selected from the group comprising SEQ ID NO: 5 (Millenium WO 98/12302), SEQ ID NO: 6 (Incyte WO 98/20117), SEQ ID NO: 7 (PNAS (1999) 96, 3114-3119), and SEQ ID NO: 8 (Corixa WO 00/04149, P703PDE5 sequence). Yet in a most preferred embodiment, the fusion protein comprises the N-terminal 81 amino acids of NS1 non structural protein fused to the 5 to 226 carboxy-terminal amino acids from mutated prostase, as set forth in SEQ ID NO: 1 or SEQ ID N°3.

The proteins of the present invention are expressed in an appropriate host cell, and preferably in *E. coli*. In a preferred embodiment the proteins are expressed with an affinity tag, such as for example, a histidine tail comprising between 5 to 9 and preferably six histidine residues, most preferably at least 4 histidine residues. These are advantageous in aiding purification through for example ion metal affinity chromatography (IMAC).

The present invention also provides a nucleic acid encoding the proteins of the present invention. Such sequences can be inserted into a suitable expression vector and used for DNA/RNA vaccination or expressed in a suitable host. In additional embodiments, genetic constructs comprising one or more of the polynucleotides of the invention are introduced into cells *in vivo*. This may be achieved using any of a variety or well-known approaches. One of the preferred methods for *in vivo* delivery of one or more nucleic acid sequences involves the use of an expression vector such as a recombinant live viral or bacterial microorganism. Suitable viral expression vectors are for example poxviruses (e.g; vaccinia, fowlpox, canarypox), alphaviruses (Sindbis virus, Semliki Forest Virus, Venezuelian Equine Encephalitis Virus), adenoviruses, adeno-associated virus, picornaviruses (poliovirus, rhinovirus), and herpesviruses (varicella zoster virus, etc). Other preferred methods for *in vivo* delivery of one or more nucleic acid sequences involves the use of a bacterial expression vector, such as Listeria, Salmonella , Shigella and BCG. Inoculation and in vivo infection with this live vector will lead to in vivo expression of the antigen and

induction of immune responses. These viruses and bacteria can be virulent, or attenuated in various ways in order to obtain live vaccines. Such live vaccines also form part of the invention.

A DNA sequence encoding the proteins of the present invention can be synthesized using standard DNA synthesis techniques, such as by enzymatic ligation as described by D.M. Roberts *et al.* in Biochemistry 1985, *24*, 5090-5098, by chemical synthesis, by *in vitro* enzymatic polymerization, or by PCR technology utilising for example a heat stable polymerase, or by a combination of these techniques. Enzymatic polymerisation of DNA may be carried out *in vitro* using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50µl or less. Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer, such as 0.05M Tris (pH 7.4), 0.01M $MgCl_2$, 0.01M dithiothreitol, 1mM spermidine, 1mM ATP and 0.1mg/ml bovine serum albumin, at a temperature of 4°C to ambient, generally in a volume of 50ml or less. The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982), or in other scientific publications, for example M.J. Gait, H.W.D. Matthes, M. Singh, B.S. Sproat, and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B.S. Sproat, and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H. Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams *et al.*, Journal of the American Chemical Society, 1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus, and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes *et al.*, EMBO Journal, 1984, 3, 801.

In a further embodiment of the invention is provided a method of producing a protein as described herein. The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis *et al.*, Molecular Cloning - A Laboratory Manual; Cold Spring Harbor, 1982-1989.

In particular, the process of the invention may preferably comprise the steps of:

i) preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes the protein or an immunogenic derivative thereof;

ii) transforming a host cell with said vector;

ii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said protein; and

iv) recovering said protein.

The term 'transforming' is used herein to mean the introduction of foreign DNA into a host cell. This can be achieved for example by transformation, transfection or infection with an appropriate plasmid or viral vector using e.g. conventional techniques as described in Genetic Engineering; Eds. S.M. Kingsman and A.J. Kingsman; Blackwell Scientific Publications; Oxford, England, 1988. The term 'transformed' or 'transformant' will hereafter apply to the resulting host cell containing and expressing the foreign gene of interest. Preferably recombinant antigens of the invention are expressed in unicellular hosts, most preferably in bacterial systems, most preferably in *E. coli*.

Preferably the recombinant strategy includes cloning a gene construct encoding a NS1 fusion protein, the gene construct comprising from 5' to 3' a DNA sequence encoding NS1 joined to a DNA sequence encoding the protein of interest, into an expression vector to form a DNA fragment encoding a NS1- carboxyl-terminal P703P fusion protein. An affinity polyhistidine tail may be engineered at the carboxy-terminus of the fusion protein allowing for simplified purification through affinity chromatography.

The expression vectors are novel and also form part of the invention.

The replicable expression vectors may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment encode the desired product, such as the DNA polymer encoding the protein of the invention, or derivative thereof, under ligating conditions.

Thus, the hybrid DNA may be pre-formed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic or eukaryotic but are preferably *E. coli*, yeast or CHO cells. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses. Expression and cloning vectors preferably contain a selectable marker such that only the host cells expressing the marker will survive under selective conditions. Selection genes include but are not limited to the one encoding protein that confer a resistance to ampicillin, tetracyclin or kanamycin. Expression vectors also contain control sequences which are compatible with the designated host. For example, expression control sequences for *E. coli*, and more generally for prokaryotes, include promoters and ribosome binding sites. Promoter sequences may be naturally occurring, such as the β-lactamase (penicillinase) (Weissman 1981, *In Interferon 3* (ed. L. Gresser), lactose (lac) (Chang et al. Nature, 1977, 198: 1056) and tryptophan (trp) (Goeddel et al. Nucl. Acids Res. 1980, 8, 4057) and lambda-derived P_L promoter system. In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. This is the case for example for the tac synthetic hybrid promoter which is derived from sequences of the trp and lac promoters (De Boer et al., Proc. Natl Acad Sci. USA 1983, 80, 21-26). These systems are particularly suitable with *E. coli*.

Yeast compatible vectors also carry markers that allow the selection of successful transformants by conferring prototrophy to auxotrophic mutants or resistance to heavy metals on wild-type strains. Control sequences for yeast vectors include promoters for glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 1968, 7, 149), PHO5 gene encoding acid phosphatase, CUP1 gene, ARG3 gene, GAL genes promoters and synthetic promoter sequences.. Other control elements useful in yeast expression are terminators and leader sequences. The leader sequence is particularly useful since it typically encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. Suitable signal sequences can be encoded by genes for secreted yeast proteins such as the yeast invertase gene and the a-factor gene, acid phosphatase, killer toxin, the a-mating factor gene and recently the heterologous inulinase signal sequence derived from INU1A gene of Kluyveromyces marxianus.. Suitable vectors have been developed for expression in *Pichia pastoris* and *Saccharomyces cerevisiae*.

A variety of *P. pastoris* expression vectors are available based on various inducible or constitutive promoters (Cereghino and Cregg, FEMS Microbiol. Rev. 2000,24:45-66). For the production of cytosolic and secreted proteins,the most commonly used P. pastoris vectors contain the very strong and tightly regulated alcohol oxidase (AOX1) promoter. The vectors also contain the *P. pastoris* histidinol dehydrogenase (HIS4) gene for selection in his4 hosts. Secretion of foreign protein require the presence of a signal sequence and the *S. cerevisiae* prepro alpha mating factor leader sequence has been widly and successfully used in Pichia expression system. Expression vectors are integrated into the P. pastoris genome to maximize the stability of expression strains. As in *S.cerevisiae*, cleavage of a *P.pastoris* expression vector within a sequence shared by the host genome (AOX1 or HIS4) stimulates homologous recombination events that efficiently target integration of the vector to that genomic locus. In general, a recombinant strain that contains multiple integrated copies of an expression cassette can yield more heterologous protein than single-copy strain. The most effective way to obtain high copy number transformants requires the transformation of Pichia recipient strain by the sphaeroplast technique (Cregg et all 1985, Mol.Cell.Biol. 5: 3376-3385) .

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis *et al.* cited above.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis *et al.* cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions depends upon the choice of the host cell to be transformed. For example, in vivo transformation using a live viral vector as the transforming agent for the polynucleotides of the invention is described above. Bacterial transformation of a host such as *E. coli* may be done by direct uptake of the polynucleotides (which may be expression vectors containing the desired sequence) after the host has been treated with a solution of $CaCl_2$ (Cohen *et al.*, Proc. Nat. Acad. Sci., 1973, *69*, 2110) or with a solution comprising a mixture of rubidium chloride (RbCl), $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-

propane-sulphonic acid, RbCl and glycerol. Transformation of lower eukaryotic organisms such as yeast cells in culture by direct uptake may be carried out by using the method of Hinnen et al (Proc. Natl. Acad. Sci. 1978, 75 : 1929-1933). Mammalian cells in culture may be transformed using the calcium phosphate co-precipitation of the vector DNA onto the cells (Graham & Van der Eb, Virology 1978, 52, 546). Other methods for introduction of polynucleotides into mammalian cells include dextran mediated transfection, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) into liposomes, and direct micro-injection of the polynucleotides into nuclei.

The invention also extends to a host cell transformed with a nucleic acid encoding the protein of the invention or a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis *et al.* and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 50°C, preferably between 25°C and 35°C, most preferably at 30°C. The incubation time may vary from a few minutes to a few hours, according to the proportion of the polypeptide in the bacterial cell, as assessed by SDS-PAGE or Western blot.

The product may be recovered by conventional methods according to the host cell and according to the localisation of the expression product (intracellular or secreted into the culture medium or into the cell periplasm). Thus, where the host cell is bacterial, such as *E. coli* it may, for example, be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Where the host cell is a yeast such as *Saccharomyces cerevisiae* or *Pichia pastoris*, the product may generally be isolated from from lysed cells or from the culture medium, and then further purified using conventional techniques. The specificity of the expression system may be assessed by western blot using an antibody directed against the polypeptide of interest.

Conventional protein isolation techniques include selective precipitation, adsorption chromatography, and affinity chromatography including a monoclonal antibody affinity column. When the proteins of the present invention are expressed

with a histidine tail (His tag), they can easily be purified by affinity chromatography using an ion metal affinity chromatography column (IMAC) column.

In a preferred embodiment of the invention the proteins of the present invention is provided with an affinity tag, such as a polyhistidine tail. In such cases the protein after the blocking step is preferably subjected to affinity chromatography. For those proteins with a polyhistidine tail, immobilised metal ion affinity chromatography (IMAC) may be performed. The metal ion, may be any suitable ion for example zinc, nickel, iron, magnesium or copper, but is preferably zinc or nickel. Preferably the IMAC buffer contains detergent, preferably a non-ionic detergent such as Tween 80, or a zwitterionic detergent such as Empigen BB, as this may result in lower levels of endotoxin in the final product.

Further chromatographic steps include for example a Q-Sepharose step that may be operated either before of after the IMAC column. Preferably the pH is in the range of 7.5 to 10, more preferably from 7.5 to 9.5, optimally between 8 and 9, ideally 8.5.

The proteins of the present invention are provided either soluble in a liquid form or in a lyophilised form, which is the preferred form. It is generally expected that each human dose will comprise 1 to 1000 µg of protein, and preferably 30-300 µg.

The present invention also provides pharmaceutical composition comprising a protein of the present invention in a pharmaceutically acceptable excipient.
A preferred vaccine composition comprises at least NS1-P703P* (SEQ ID N°1) or NS1-P703P (SEQ ID N°3). Said protein has, preferably, blocked thiol groups and is highly purified, e.g. has less than 5% host cell contamination. Such vaccine may optionally contain one or more other tumour-associated antigen and derivatives. For example, suitable other associated antigen include PAP-1, PSA (prostate specific antigen), PSMA (prostate-specific membrane antigen), PSCA (Prostate Stem Cell Antigen), STEAP.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds. Powell M.F. & Newman M.J). (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The proteins of the present invention are preferably adjuvanted in the vaccine formulation of the invention. Suitable adjuvants are commercially available such as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham, Philadelphia, PA); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF or interleukin-2, -7, or -12, may also be used as adjuvants.

In the formulations of the invention it is preferred that the adjuvant composition induces an immune response predominantly of the TH1 type. High levels of Th1-type cytokines (*e.g.*, IFN-γ, TNFα, IL-2 and IL-12) tend to favour the induction of cell mediated immune responses to an administered antigen. Within a preferred embodiment, in which a response is predominantly Th1-type, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using standard assays. For a review of the families of cytokines, see Mosmann and Coffman, *Ann. Rev. Immunol.* 7:145-173, 1989.

Accordingly, suitable adjuvants for use in eliciting a predominantly Th1-type response include, for example a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt. Other known adjuvants which preferentially induce a TH1 type immune response include CpG containing oligonucleotides. The oligonucleotides are characterised in that the CpG dinucleotide is unmethylated. Such oligonucleotides are well known and are described in, for example WO 96/02555. Immunostimulatory DNA sequences are also described, for example, by Sato et al., *Science 273*:352, 1996. Another preferred adjuvant is a saponin, preferably QS21 (Aquila Biopharmaceuticals Inc., Framingham, MA), which may be used alone or in combination with other adjuvants. For example, an enhanced system involves the combination of a monophosphoryl lipid A and saponin derivative, such as the combination of QS21 and 3D-MPL as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other preferred

formulations comprise an oil-in-water emulsion and tocopherol. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil-in-water emulsion is described in WO 95/17210.

A particularly potent adjuvant formulation involving QS21 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is a preferred formulation.

Other preferred adjuvants include Montanide ISA 720 (Seppic, France), SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), Detox (Ribi, Hamilton, MT), RC-529 (Corixa, Hamilton, MT) and other aminoalkyl glucosaminide 4-phosphates (AGPs).

Other preferred adjuvants include adjuvant molecules of the general formula (I):

$HO(CH_2CH_2O)_n$-A-R

Wherein, n is 1-50, A is a bond or –C(O)-, R is C_{1-50} alkyl or Phenyl C_{1-50} alkyl.

One embodiment of the present invention consists of a vaccine formulation comprising a polyoxyethylene ether of general formula (I), wherein *n* is between 1 and 50, preferably 4-24, most preferably 9; the *R* component is C_{1-50}, preferably C_4-C_{20} alkyl and most preferably C_{12} alkyl, and *A* is a bond. The concentration of the polyoxyethylene ethers should be in the range 0.1-20%, preferably from 0.1-10%, and most preferably in the range 0.1-1%. Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether, polyoxyethylene-9-steoryl ether, polyoxyethylene-8-steoryl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether. Polyoxyethylene ethers such as polyoxyethylene lauryl ether are described in the Merck index (12^{th} edition: entry 7717). These adjuvant molecules are described in WO 99/52549.

The polyoxyethylene ether according to the general formula (I) above may, if desired, be combined with another adjuvant. For example, a preferred adjuvant combination is preferably with CpG as described in the pending UK patent application GB 9820956.2.

Accordingly in one embodiment of the present invention there is provided a vaccine comprising a protein of the present invention, more preferably a NS1-P703P*

adjuvanted with a monophosphoryl lipid A or derivative thereof, QS21 and tocopherol in an oil in water emulsion.

Preferably the vaccine additionally comprises a saponin, more preferably QS21. Another particular suitable adjuvant formulation including CpG and a saponin is described in WO 00/09159 and is a preferred formulation. Most preferably the saponin in that particular formulation is QS21. Preferably the formulation additionally comprises an oil in water emulsion and tocopherol.

Any of a variety of delivery vehicles may be employed within pharmaceutical compositions and vaccines to facilitate production of an antigen-specific immune response that targets tumour cells. Delivery vehicles include antigen-presenting cells (APCs), such as dendritic cells, macrophages, B cells, monocytes and other cells that may be engineered to be efficient APCs. Such cells may, but need not, be genetically modified to increase the capacity for presenting the antigen, to improve activation and/or maintenance of the T cell response, to have anti-tumour effects *per se* and/or to be immunologically compatible with the receiver (*i.e.*, matched HLA haplotype). APCs may generally be isolated from any of a variety of biological fluids and organs, including tumour and peri-tumoural tissues, and may be autologous, allogeneic, syngeneic or xenogeneic cells.

Certain preferred embodiments of the present invention use dendritic cells or progenitors thereof as antigen-presenting cells. Dendritic cells are highly potent APCs (Banchereau and Steinman, *Nature 392*:245-251, 1998) and have been shown to be effective as a physiological adjuvant for eliciting prophylactic or therapeutic antitumour immunity (*see* Timmerman and Levy, *Ann. Rev. Med. 50*:507-529, 1999). In general, dendritic cells may be identified based on their typical shape (stellate *in situ*, with marked cytoplasmic processes (dendrites) visible *in vitro*), their ability to take up, process and present antigens with high efficiency and their ability to activate naïve T cell responses. Dendritic cells may, of course, be engineered to express specific cell-surface receptors or ligands that are not commonly found on dendritic cells *in vivo* or *ex vivo*, and such modified dendritic cells are contemplated by the present invention. As an alternative to dendritic cells, secreted vesicles antigen-loaded dendritic cells (called exosomes) may be used within a vaccine (*see* Zitvogel et al., *Nature Med. 4*:594-600, 1998).

Dendritic cells and progenitors may be obtained from peripheral blood, bone marrow, tumour-infiltrating cells, peritumoral tissues-infiltrating cells, lymph nodes, spleen, skin, umbilical cord blood or any other suitable tissue or fluid. For example, dendritic cells may be differentiated *ex vivo* by adding a combination of cytokines such as GM-CSF, IL-4, IL-13 and/or TNFα to cultures of monocytes harvested from peripheral blood. Alternatively, CD34 positive cells harvested from peripheral blood, umbilical cord blood or bone marrow may be differentiated into dendritic cells by adding to the culture medium combinations of GM-CSF, IL-3, TNFα, CD40 ligand, lipopolysaccharide LPS, flt3 ligand and/or other compound(s) that induce differentiation, maturation and proliferation of dendritic cells.

Dendritic cells are conveniently categorized as "immature" and "mature" cells, which allows a simple way to discriminate between two well characterized phenotypes. However, this nomenclature should not be construed to exclude all possible intermediate stages of differentiation. Immature dendritic cells are characterized as APC with a high capacity for antigen uptake and processing, which correlates with the high expression of Fcγ receptor and mannose receptor. The mature phenotype is typically characterized by a lower expression of these markers, but a high expression of cell surface molecules responsible for T cell activation such as class I and class II MHC, adhesion molecules (*e.g.*, CD54 and CD11) and costimulatory molecules (*e.g.*, CD40, CD80, CD86 and 4-1BB).

APCs may generally be transfected with a polynucleotide encoding prostase tumour protein (or derivative thereof) such that the prostase tumour polypeptide, or an immunogenic portion thereof, is expressed on the cell surface. Such transfection may take place *ex vivo*, and a composition or vaccine comprising such transfected cells may then be used for therapeutic purposes, as described herein. Alternatively, a gene delivery vehicle that targets a dendritic or other antigen presenting cell may be administered to a patient, resulting in transfection that occurs *in vivo*. *In vivo* and *ex vivo* transfection of dendritic cells, for example, may generally be performed using any methods known in the art, such as those described in WO 97/24447, or the gene gun approach described by Mahvi et al., *Immunology and cell Biology 75*:456-460, 1997. Antigen loading of dendritic cells may be achieved by incubating dendritic cells or progenitor cells with the prostase tumour polypeptide, DNA (naked or within

a plasmid vector) or RNA; or with antigen-expressing recombinant bacterium or viruses (*e.g.*, vaccinia, fowlpox, adenovirus or lentivirus vectors).

Vaccines and pharmaceutical compositions may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are preferably hermetically sealed to preserve sterility of the formulation until use. In general, formulations may be stored as suspensions, solutions or emulsions in oily or aqueous vehicles. Alternatively, a vaccine or pharmaceutical composition may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier immediately prior to use.

The present invention also provides a method for producing a vaccine formulation comprising mixing a protein of the present invention together with a pharmaceutically acceptable excipient, such as 3D-MPL.

Another aspect of the invention is the use of a protein or nucleic acid as claimed herein for the manufacture of a vaccine for immunotherapeutically treating a patient suffering from prostate cancer or other prostase-associated tumours.

FIGURES LEGENDS

Figure 1: Design of the fusion protein NS1- p703* -His expressed in *E. coli*

Figure 2: Primary structure of the fusion protein NS1- p703* -His expressed in *E. coli* (SEQ ID N°1).

Figure 3: Coding sequence of $NS1_{1-81}$-P703P*-His (SEQ ID N°2).

Figure 4: Cloning strategy to produce NS1-P703P*-His in *E. coli*

Figure 5: Plasmid map of RIT 14952

Figure 6: *E. coli* NS1-P703P*-His fermentation process

Figure 7: *E. coli* NS1-P703P*-His purification process

Figure 8: Immunogenicity of NS1-P703P* adjuvanted with SBAS2

Figure 9: Primary structure of the fusion protein NS1- p703 -His expressed in *E. coli* (SEQ ID N°3).

Figure 10: Coding sequence of $NS1_{1-81}$-P703P-His (SEQ ID N°4).

The invention will be further described by reference to the following examples:

EXAMPLE I:

Preparation of the recombinant *E. coli* strain expressing the fusion protein NS1-P703P*-3-His

1. – Protein design

The expression strategy followed for this candidate included the design of the most appropriate primary structure for the recombinant protein that could have the best expectation for both, good level of expression and easy purification process.

Although the chance that a recombinant protein could keep its protease biological activity when formulated for vaccination is really very low, the mutation of the active side was made in order to reduce substantially or preferably eliminate its proteolytical biological activity. Accordingly, the His residue at position 43 of SEQ ID N° 8, has been mutated into an Ala residue.

The design of the fusion protein NS1- p703* -His to be expressed in *E. coli* is described in figure 1. This fusion contains the N-terminal (81 amino acid) of non structural protein of Influenzae virus, followed by the non processed amino acid sequence of prostate antigen (amino acids 5→226 of p703pde5 sequence described in SEQ ID N°8 containing the mutation His→Ala of the 43 residue of the protease active site followed by the His tail. The Histidine tail was added to prostase to enable versatile purification of the fusion and processed protein. The length of the fusion is 313 aminoacids.

The primary structure of the resulting protein has the sequence described in figure 2. The coding sequence corresponding to the above protein is illustrated in figure 3 and was subsequently placed under the control of λpL promoter in a *E. coli* expression plasmid.

2. – The *E. Coli* expression system

For the production of NS1 the DNA encoding the 81 amino-terminal residues of NS1 (non-structural protein from influenzae virus) has been cloned into the expression vector pMG 81. This plasmid utilises signals from lambda phage DNA to drive the transcription and translation of inserted foreign genes. The vector contains the lambda PL promoter PL, operator OL and two utilisation sites (NutL and NutR) to relieve transcriptional polarity effects when N protein is provided (Gross et al., 1985. Mol. & Cell. Biol. 5:1015). Vectors containing the PL promoter, are introduced into an E. coli lysogenic host to stabilise the plasmid DNA. Lysogenic host strains contain replication-defective lambda phage DNA integrated into the genome (Shatzman et al., 1983; In Experimental Manipulation of Gene Expression. Inouya (ed) pp 1-14. Academic Press NY). The lambda phage DNA directs the synthesis of the cI repressor protein which binds to the OL repressor of the vector and prevents binding of RNA polymerase to the PL promoter and thereby transcription of the inserted gene. The cI gene of the expression strain AR58 contains a temperature sensitive mutation so that PL directed transcription can be regulated by temperature shift, i.e. an increase in culture temperature inactivates the repressor and synthesis of the foreign protein is initiated. This expression system allows controlled synthesis of foreign proteins especially of those that may be toxic to the cell (Shimataka & Rosenberg, 1981. Nature 292:128).

3. – The *E. Coli* strain AR58:

The AR58 lysogenic E. coli strain used for the production of the NS1-P703P*-His protein is a derivative of the standard NIH E.coli K12 strain N99 (F- su- galK2, lacZ- thr-). It contains a defective lysogenic lambda phage (galE::TN10, l Kil- cI857 DH1). The Kil- phenotype prevents the shut off of host macromolecular synthesis. The cI857 mutation confers a temperature sensitive lesion to the cI repressor. The DH1 deletion removes the lambda phage right operon and the hosts bio, uvr3, and chlA loci. The AR58 strain was generated by transduction of N99 with a P lambda phage stock previously grown on an SA500 derivative (galE::TN10, l Kil- cI857 DH1). The introduction of the defective lysogen into N99 was selected with tetracycline by virtue of the presence of a TN10 transposon coding for tetracyclin

resistance in the adjacent galE gene. N99 and SA500 are *E.coli* K12 strains derived from Dr. Martin Rosenberg's laboratory at the National Institutes of Health.

4. – Construction of the vector designed to express the recombinant protein NS1-P703P*-His

The starting materials were:

1) A cDNA plasmid received from CORIXA p703pde5 (WO 00/04149), where the putative signal sequence and a piece of the pro-peptide of P703P is missing (see fig 1) and containing the coding sequence for prostase antigen;

2) Vector pRIT14901 containing the long version of the PL promoter; and

3) Plasmid PMG81 containing the 81aa of NS_1 coding region from Influenzae virus.

The cloning strategy outlined in figure 4 included the following steps:

a) PCR amplification of the p703 sequence with NcoI and SpeI restriction sites
The template for the PCR reaction was the cDNA plasmid received from CORIXA, the oligonucleotide sense can139 : 5'GCG CCC ATG GTT GGG GAG GAC TGC AGC CCG 3', and the oligonucleotide antisense can134 : 5'GGG ACT AGT ACT GGC CTG GAC GGT TTT CTC 3';

b) Insertion of the amplified sequences into the commercial vector Litmus 28 (biolabs), leading to the intermediate plasmid pRIT 14949;

c) Directed His → Ala mutagenesis of residue 43 of the p703 sequence contained in the plasmid pRIT 14949, using the sense oligonucleotide can140 : 5'CTG TCA GCC GCA GCG TGT TTC CAG 3'and the antisense oligonucleotide can141 : 5' CTG GAA ACA CGC TGC GGC TGA CAG 3', leading to the obtention of plasmid pRIT 14950;

d) Isolation of the NcoI - SpeI fragment from the plasmid pRIT 14950;

e) From pMG81 plasmid, purification of NS1 fragment (81aa) after digestion of the restriction sites BamHI – NcoI;

f) Ligation of both fragments were ligated to the expression plasmid pRIT 14901 (pr PL long);

g) Selection and characterisation of the *E. coli* AR58 strain transformants containing the plasmid pRIT14952 (see figure 5) expressing the NS1- p703 mutated -His fusion protein

The recombinant strain thus produces the NS1-P703P* His-tailed fusion protein of 313 amino acid residues long (see Figure 2), with the amino acids sequence described in ID No1 and the coding sequence is described in ID No2.

EXAMPLE II:

Preparation of the recombinant NS1-P703P*-3-His fusion protein

1. – Growth and induction of bacterial strain B1225 - Expression of NS1-P703P*-3-His

Cells of AR58 transformed with plasmid pRIT14952 (strain B1225) were grown in a 2 L flask containing 400 ml FEC015AA medium supplemented with kanamycin sulphate (100mg/L). After a 16h of incubation at 30°C and at 200 rpm, a small sample was removed from this flask for microscopic examination.

50 ml of this pre-culture was transferred into a 20-L fermentor containing 8.7 L of FEC012AF medium supplemented with kanamycin sulphate (50mg/L). The pH was adjusted to and maintained at 6.8 by addition of NH4OH (25 % v/v), and the temperature was maintained at 30°C. The aeration rate was kept constant at 20 L/min and the pO2 was regulated at 20% of saturation by feedback control of the agitation speed. The head pressure was maintained at 0.5 bar.

This fed-batch fermentation process is based on glycerol as a carbon source. The feed solution was added at an initial rate of 0.04ml/min, and increased exponentially during the first 30 hours to limit the growth rate in order to be able to keep a minimum pO2 level of 20%.

After 30 hours, the temperature of the fermentor was rapidly increased to 39.5°C in order to induce the intracellular expression of the antigen NS1-P703P*-His. The feeding rate was maintained constant at 1.28 ml/min during the whole induction phase (18h).

Samples of broth were taken during both growth and induction phases in order to monitor bacterial growth and antigen expression. Microbiological identification and purity tests were also realised on these materials.

At the end of fermentation, the biomass reached an optical density of about 130, corresponding to a dry cell weight of about 50g/L. The final volume was approximately 10.5 L. The cells containing the antigen were directly separated from the culture medium by centrifugation at 5000g for 1 h at 4°C and the pellet was stored in plastic bags at –70°C.

2. – Extraction of the protein:

Recombinant NS1-P703P*-His protein, expressed in *E. coli* as inclusion bodies, was purified from cell homogenate using different steps (see figure 6).
Briefly, frozen concentrated cells from fermentation harvest were thawed to +4°C before being resuspended in disruption buffer (phosphate 20 mM - NaCl 2M - EDTA 5 mM pH 7.5) to a final optical density (OD650) of 120. Two passes through a high-pressure homogeniser (1000 bars) disrupted the cells.

EXAMPLE III:

Purification of fusion Protein NS1-P703P*-His

a) Introduction

As said above, the recombinant protein, NS1-P703P*-His is produced in *E. coli* in the form of inclusion bodies. A major issue for the set-up of the purification method was the oxidation of the recombinant protein with itself or with host cell contaminants, likely through covalent binding with disulphide bonds. The process as developed aimed at reducing the massive oxidation phenomenon in order to have a highly purified product together with an acceptable global yield, while preserving the product ability to mount an effective immune response against the antigen of interest.

a) Description of the process

The broken cell suspension was treated on a Pallsep VMF (Vibrating Membrane Filtration) system (Pall-Filtron) equipped with 0.45 µm membrane. The "pellet fraction" was first washed by diafiltration with 20 mM phosphate buffer pH 7.5 containing 0.5% Empigen BB detergent. The washed material was then solubilised in the same buffer containing 4M guanidine hydrochoride and 20 mM glutathion. The product was recovered through 0.45 µm filter and the permeate was treated with 200 mM iodoacetamide to prevent oxidative re-coupling.

The carboxyamidated fraction was subjected to IMAC (Nickel-Chelating-Sepharose FF, Pharmacia). The column was first equilibrated with 20 mM Tris buffer pH 7.5 containing 4M urea, 0.5% Tween 80, and 20 mM imidazole. After the sample loading, the column was washed with the same buffer. The protein was then eluted in the previous buffer with 400 mM Imidazole.

Before continuing the anion exchange chromatography, the conductance of the IMAC-eluate was reduced to below 5 mS/cm with 20 mM Tris buffer pH 8.5 containing 4M urea and 0.5-1.0 % Tween 80. The packed bed support (Q-Sepharose FF, Pharmacia) was equilibrated with the dilution buffer. After the sample loading and a washing step with the equilibration buffer, the protein was eluted with the same buffer containing 250 mM NaCl.

The Q-Sepharose FF-eluate was then diafiltered against the appropriate storage buffer (20 mM Tris buffer pH 8.0) in a tangential flow filtration unit equipped with a 10 Kd cut-off membrane (Omega, Filton).

Ultrafiltration retentate containing NS1-P703P*-His was sterile filtered through 0.22 µm membrane.

The global purification yield was very high: around 3-4 g of purified material / L of homogenate (DO120).

EXAMPLE IV:

Vaccine preparation using NS1-P703P*-His protein

1. – Vaccine preparation using NS1-P703P*-His protein:

The vaccine used in these experiments is produced from a recombinant DNA, encoding a NS1-P703P*-His, expressed in *E. coli* from the strain AR58, either adjuvanted or not. As an adjuvant, the formulation comprises a mixture of 3 de -O-acylated monophosphoryl lipid A (3D-MPL) and QS21 in an oil/water emulsion. The adjuvant system SBAS2 has been previously described WO 95/17210.

3D-MPL: is an immunostimulant derived from the lipopolysaccharide (LPS) of the Gram-negative bacterium *Salmonella minnesota*. MPL has been deacylated and is lacking a phosphate group on the lipid A moiety. This chemical treatment dramatically reduces toxicity while preserving the immunostimulant properties (Ribi, 1986). Ribi Immunochemistry produces and supplies MPL to SB-Biologicals.
Experiments performed at Smith Kline Beecham Biologicals have shown that 3D-MPL combined with various vehicles strongly enhances both the humoral and a TH1 type of cellular immunity.

QS21: is a natural saponin molecule extracted from the bark of the South American tree Quillaja saponaria Molina. A purification technique developed to separate the individual saponines from the crude extracts of the bark, permitted the isolation of the particular saponin, QS21, which is a triterpene glycoside demonstrating stronger adjuvant activity and lower toxicity as compared with the parent component. QS21 has been shown to activate MHC class I restricted CTLs to several subunit Ags, as well as to stimulate Ag specific lymphocytic proliferation (Kensil, 1992). Aquila (formally Cambridge Biotech Corporation) produces and supplies QS21 to SB-Biologicals.

Experiments performed at SmithKline Beecham Biologicals have demonstrated a clear synergistic effect of combinations of MPL and QS21 in the induction of both humoral and TH1 type cellular immune responses.

The oil/water emulsion is composed an organic phase made of of 2 oils (a tocopherol and squalene), and an aqueous phase of PBS containing Tween 80 as emulsifier. The emulsion comprised 5% squalene 5% tocopherol 0.4% Tween 80 and had an average particle size of 180 nm and is known as SB62 (see WO 95/17210).

Experiments performed at SmithKline Beecham Biologicals have proven that the adjunction of this O/W emulsion to 3D-MPL/QS21 (SBAS2) further increases the immunostimulant properties of the latter against various subunit antigens.

2. – Preparation of emulsion SB62 (2 fold concentrate):

Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100 ml two fold concentrate emulsion 5g of DL alpha tocopherol and 5ml of squalene are vortexed to mix thoroughly. 90ml of PBS/Tween solution is added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M110S microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

3. – Lyophilisation of NS1-P703P*-His:

In practice, all compounds are placed in solution and sterilisation is achieved by filtration on a 0.2µm membrane. Formulations were performed the day of freeze-drying.

The sequence of formulation was:

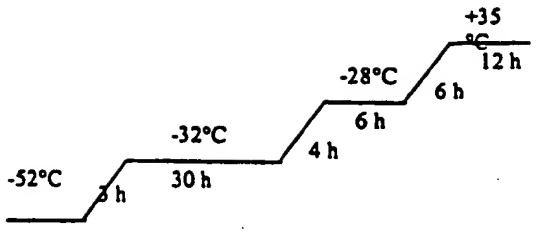
H_2O + sucrose 15.75% + Tris 100 mM pH8 + Tween80 10%- $_{5\ min}$ + NS1-P703p

The volumes of all compounds are adjusted to have in final:

250-50-10 µg NS1-P703P*-His, in Tris 10 mM, tween 8O 0.2%, 3.15% sucrose.

The vial was overfilled with by1.25 x (reconstitution with 625 µl diluant, injection of 500µl).

Using the Lyovac GT6 lyophilisation apparatus purchased from Steris (Germany), the lyophilisation cycle was performed during 3 days as follows:



4. – Preparation of NS1-P703P*-His QS21/3D MPL oil in water (SBAS2) formulation:

The adjuvant is formulated as a combination of MPL and QS21, in an oil/water emulsion.

1) Formulation composition (injection volume: 100µl); group 1 has received P703P*-His (20 µg) formulated in a combination of MPL and QS21, in an oil/water emulsion. Group 2 has received NS1-P703P*-His (25 µg) in a combination of MPL and QS21, in an oil/water emulsion.

2) Components

Components	Conc mg/ml	Buffer
P703p-His	0.513	Po4 20mM pH 7.5
NS1-P703p-his carboxy	0.846	Tris 20 mM Tween 80 0.2% pH7.5
SB62	2 x	PBS pH 6.8
MPL	8.175	H_2O
QS21	2	H_2O
Thiomersal	0.2	H_2O

3) Formulations

The formulations were prepared extemporaneously on the day of injection.

The formulations containing 3D-MPL and QS21 in an oil/water emulsion (SBAS2B formulations - Groups 2 and 3) were performed as follows: P703p (20µg) (group 2) and NS1-P703P*-His (25µg) (group 3) were diluted in 10-fold concentrated PBS pH 6.8 and H_2O before consecutive addition of SB62 (50µl), MPL (20µg), QS21 (20µg) and 1 µg/ml thiomersal as preservative at 5 min intervals. All incubations were carried out at room temperature with agitation.

The non-adjuvanted formulations (Groups 4 and 5) were performed as follows: P703p (20µg) (group 4) and NS1-P703P*-His (25µg) (group 5) were diluted in 1.5 M NaCl and H_2O before addition of 1 µg/ml thiomersal as preservative at 5 min intervals. All incubations were carried out at room temperature with agitation.

The final vaccine is obtained after reconstitution of the lyophilised NS1-P703P*-His preparation with the adjuvant or with PBS alone.

The adjuvant controls without antigen were prepared by replacing the protein by PBS.

EXAMPLE V:

Immunogenicity using NS1-P703P*-His protein

1. – Immunogenicity of NS1-P703P*-His in mice

The aim of the experiment was to characterise the immune response induced in mice by vaccination with the purified recombinant mutated NS1-p703*-His molecule produced in *E coli*, in the presence or the absence of an adjuvant.

a) - Immunization protocol:

Groups of 10 immunocompetent Balb/c mice, 6 to 8 weeks old mice, were vaccinated twice, intramuscularly, at 2 weeks interval with 25 µg of mutated NS1-P703 formulated or not in SB AS02B (50µl SB62 / 10µg MPL / 10µg QS21).

14 days after the second injection, blood was taken and the sera were tested for the presence of anti-P703 antibodies.

b) – Total IgG Antibody response:

The anti P703 antibody response has been assessed in the sera of the mice 14 days after the latest vaccination. This has been done by ELISA using NS1-P703P* as coating antigen.

E coli extracts were used to check for the possible presence of antibodies against host contaminants.

c) – Results:

The results show that 1) a higher immune response (IgG1) is induced by NS1-P703P* as evidenced in the sera of mice injected with the NS1-P703P* protein alone as compared to the sera of normal control mice; 2) high antibody titers are found in animals receiving the NS1-P703P* molecule formulated in the AS02B adjuvant.

The isotypic profile of the NS1-p703p specific IgG response has also been measured. As shown in Figure 8, IgG1 were detected when mice received the NS1-P703P* protein alone, however the isotypic profile was pushed towards a TH1 response (more IgG2a) by the presence of the AS02 adjuvant.

EXAMPLE VI:

1. – NS1-P703P-His

In an analogous fashion NS1-P703P-His was prepared. The amino acid and DNA sequences are depicted in SEQUENCE ID Nos. 3 and 4.

Briefly, the strategy to express a NS1-P703P-His fusion protein in *E.coli* included the following steps:

a) As a starting materiel, the same starting material as described in Example I (amino acids 5→226 of p703pde5 sequence described in SEQ ID N°8);
b) PCR amplification to flank the p703 unmutated sequence cloning restriction sites;
c) Insertion in a PMG81 vector (promoter pL long) containing the NS1 gene;

d) Transformation of the recipient strain AR58 or AR120

e) Selection of recombinant strain.

The resulting protein can be purified in an analogous manner to the NS1-P703P*-His mutated protein. The primary structure of the resulting protein has the sequence described in figure 9. The coding sequence corresponding to the above protein is illustrated in figure 10.

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- Xue BH., Zhang Y., Sosman J. et al. "Induction of Human Cytotoxic T-Lymphocytes Specific for Prostate-Specific Antigen." *Prostate* 30(2):73-78 (1997)

CLAIMS:

1. A prostase protein or a derivative thereof linked to a fusion partner.

2. A protein as claimed in claim 1 wherein the prostase antigen is selected from the group consisting of SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7, and SEQ ID N° 8.

3. A protein as claimed in claim 1 or 2 wherein the fusion partner is an immunological fusion partner or an expression enhancer fusion partner.

4. A protein as claimed in claim 1 to 3 wherein the fusion partner is NS1 protein from influenza or a fragment thereof.

5. An antigen as claimed in any of claims 1 to 4 wherein the fusion protein further comprises an affinity tag.

6. A nucleic acid sequence encoding a protein as claimed in any of claims 1 to 5.

7. An expression vector containing a nucleic acid of claim 6.

8. A host cell transformed with a nucleic acid sequence of claim 6 or with a vector as claimed in claim 7.

9. A vaccine containing a protein as claimed in any of claims 1 to 5 or a nucleic acid as claimed in claim 6.

10. A vaccine as claimed in claim 9 additionally comprising an adjuvant, and/or immunostimulatory cytokine or chemokine.

11. A vaccine as claimed in claim 9 or 10 wherein the protein is presented in an oil in water or a water in oil emulsion vehicle.

12. A vaccine as claimed in claim 10 or 11 wherein the adjuvant comprises 3D-MPL, QS21, a CpG oligonucleotide or a polyethylene ether or ester.

13. A vaccine as claimed in any of claims 9 to 12 additionally comprising one or more other antigens.

14. A vaccine as claimed herein for use in medicine.

15. Use of a protein or nucleic acid as claimed herein for the manufacture of a vaccine for immunotherapeutically treating a patient suffering from prostate cancer or other prostase-associated tumours.

16. A process for the production of a protein as claimed in any of claims 1 to 5 comprising transforming a host cell with a nucleic acid sequence of claim 6, expressing said sequence and isolating the desired product.

17. A process for the production of a vaccine, comprising the steps of purifying a prostase protein or a derivative thereof, by the process of claim 16 and admixing the resulting protein as claimed herein with a suitable adjuvant, diluent or other pharmaceutically acceptable excipient.

18. A method of treating patients susceptible to or suffering from prostate-cancer comprising administering to said patients a pharmaceutically active amount of the vaccine according to claims 9 to 14.

Fig. 1: Design of the fusion protein NS1- p703* -His expressed in *E. coli*.

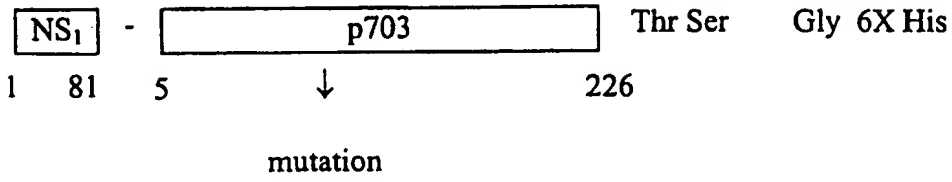

Fig. 2: Primary sequence of the NS1- p703* -His protein expressed in *E. coli*

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Fig. 3: Nucleotide sequence of NS_1-p703 mutated-His (pRIT14952)

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Fig. 4 cloning strategy to produce NS1-P703P*-His in E. coli

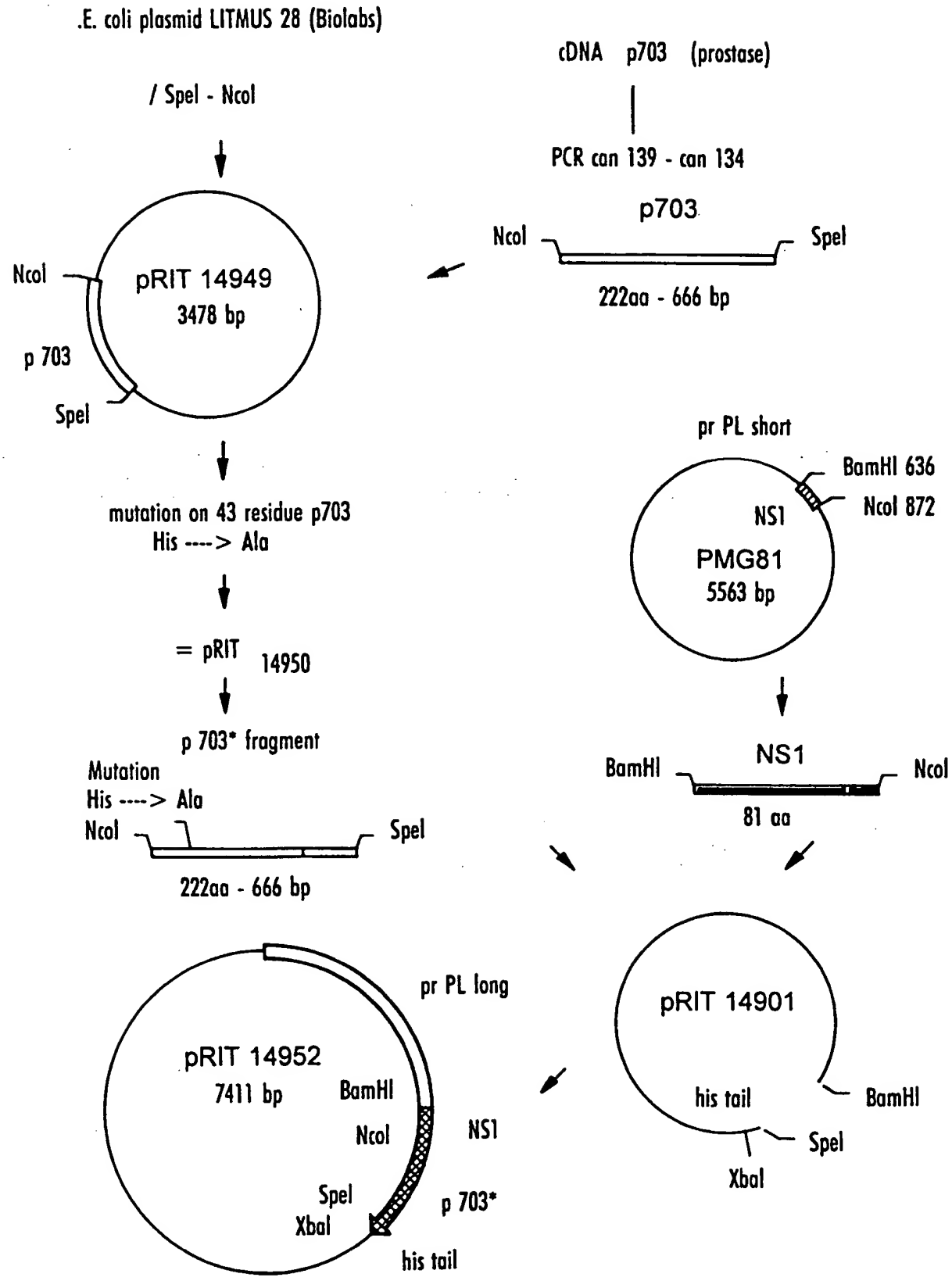

Fig. 5 Plasmid map of pRIT 14952

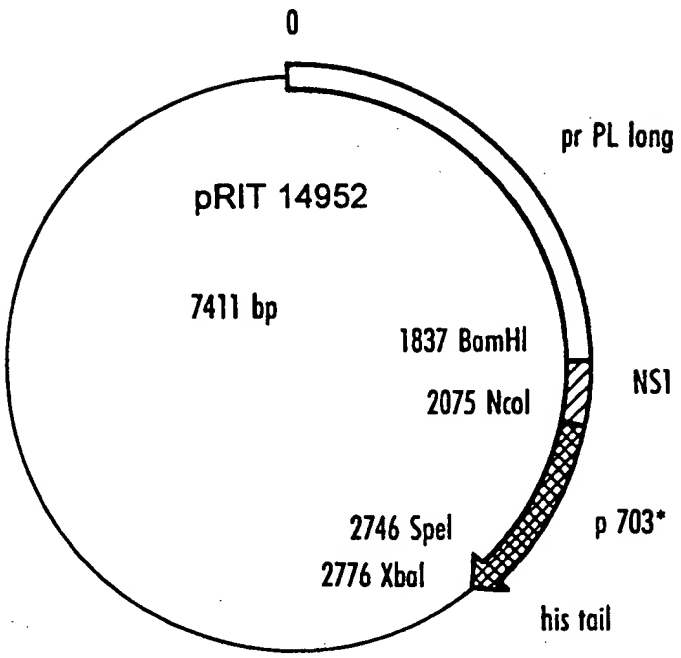

Fig. 6: E. coli NS1-P703P*-His fermentation process

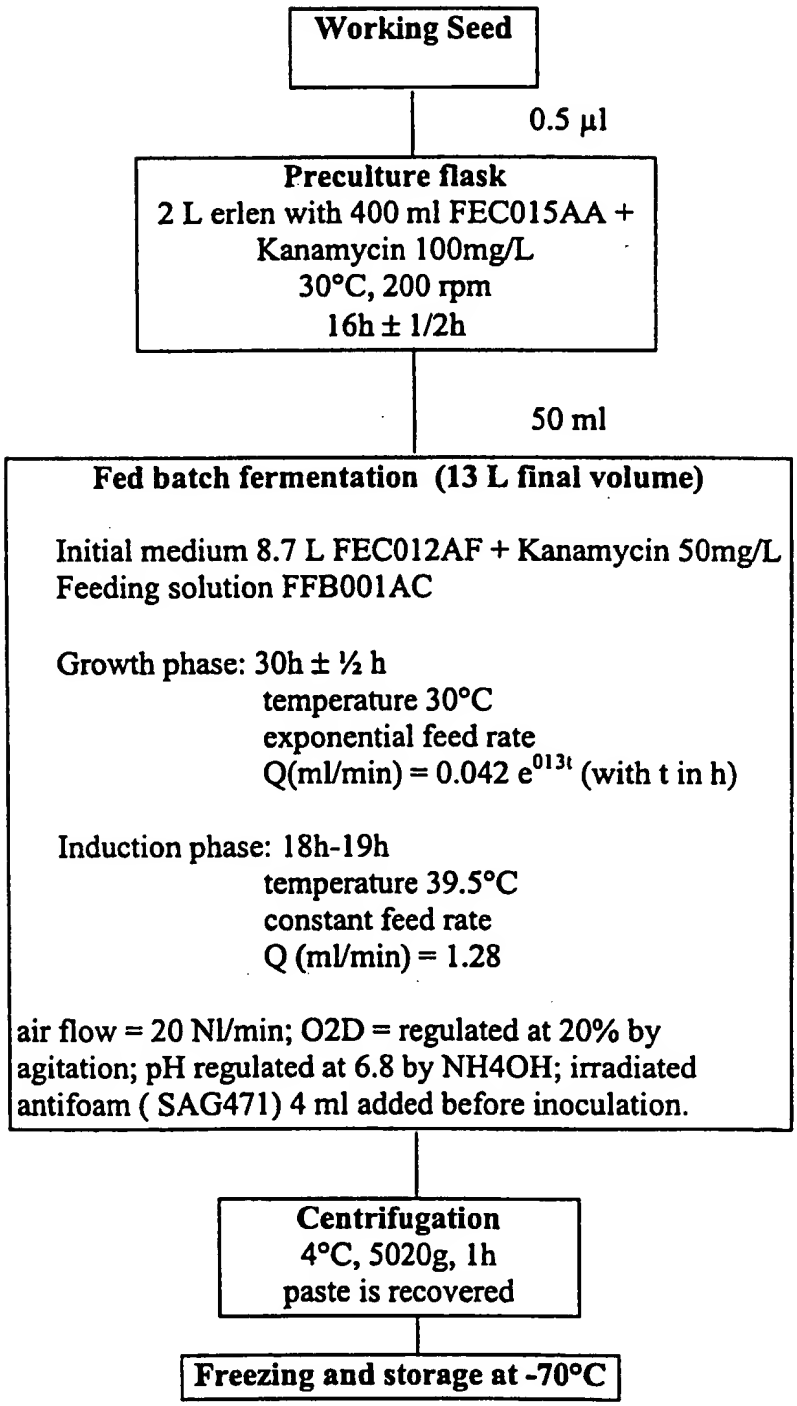

Fig. 7 : E. coli NS1-P703P*-His purification process

Frozen cells

⇓

Rannie homogenization — DO 120, 2 passes, 15 000 psi
P 20 - NaCl 2M - EDTA 5mM pH 7.5

⇓

VMF
(0.45 µm membrane) — **Pellet washing:**
P 20 – Empi 0.5% pH 7,5
Solubilization / Reduction:
Id. + GuHCl 4M – Glutathion 20 mM
Microfiltration

⇓

Carboxyamidation — 15', RT, + 200 mM Iodoacetamide

⇓

Ni-chelating sepharose (+) — Loading:
Tris 20 - Urea 4M - Tween 0.5% - Im 20mM pH 7.5
Elution:
Tris 20 - Urea 4M - Tween 0.5% - Im 400mM pH 7.5

⇓

Q-sepharose FF (+) — Dilution and Loading:
Tris 20 - Urea 4M - Tween 0.5-1.0% pH 8.5
Elution:
Id. + NaCl 250mM

⇓

Ultrafiltration
(10 Kd membrane) — **(Concentration → 1000 µg/ml)**
Diafiltration:
Tris 20 pH 8.0

⇓

Sterile filtration
(0,22 µm membrane)

Fig. 8 Immunogenicity of NS1-P703P* adjuvanted with SBAS2

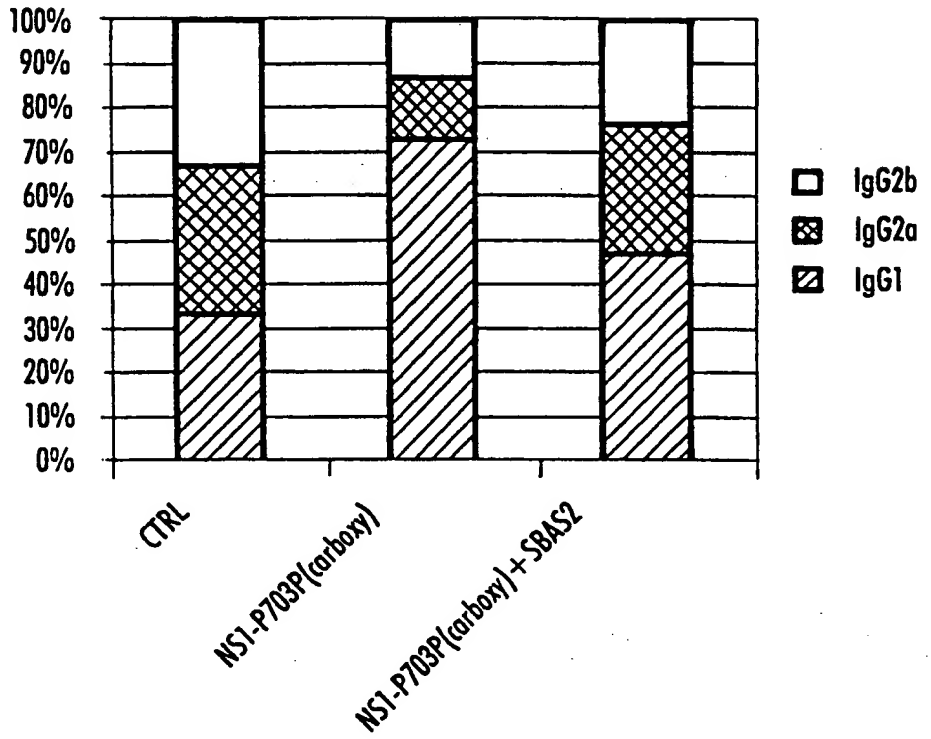

Fig. 9: Primary sequence of the NS1- p703 -His protein expressed in *E. coli*

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Fig. 10: Nucleotide sequence of NS_1-p703 -His

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            260                 265                 270
Gly Lys Ala Pro Cys Gly Gln Val Gly Val Pro Gly Val Tyr Thr Asn
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1 5 10 15
Glu Asn Glu Leu Phe Cys Ser Gly Val Leu Val His Pro Gln Trp Val
20 25 30
Leu Ser Ala Ala His Cys Phe Gln Lys Xaa Val Gln Ser Ser Tyr Thr
35 40 45
Ile Gly Leu Gly Leu His Ser Leu Glu Ala Asp Gln Glu Pro Gly Ser
50 55 60
Gln Met Val Glu Ala Ser Leu Ser Val Arg His Pro Glu Tyr Asn Arg
65 70 75 80
Pro Leu Leu Ala Asn Asp Leu Met Leu Ile Lys Leu Asp Glu Ser Val
85 90 95
Ser Glu Ser Asp Thr Ile Arg Ser Ile Ser Ile Ala Ser Gln Cys Pro

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            100                 105                 110
Thr Ala Gly Asn Ser Cys Leu Val Ser Gly Trp Gly Leu Leu Ala Asn
        115                 120                 125
Gly Arg Met Pro Thr Val Leu Gln Cys Val Asn Val Ser Val Val Ser
    130                 135                 140
Glu Glu Val Cys Ser Lys Leu Tyr Asp Pro Leu Tyr His Pro Ser Met
145                 150                 155                 160
Phe Cys Ala Gly Gly Gly Gln Asp Gln Lys Asp Ser Cys Asn Gly Asp
                165                 170                 175
Ser Gly Gly Pro Leu Ile Cys Asn Gly Tyr Leu Gln Gly Leu Val Ser
            180                 185                 190
Phe Gly Lys Ala Pro Cys Gly Gln Val Gly Val Pro Gly Val Tyr Thr
        195                 200                 205
Asn Leu Cys Lys Phe Thr Glu Trp Ile Glu Lys Thr Val Pro Gly Gln
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Leu Thr Leu Gly Thr Gly Asn Pro
225                 230
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<211> 248
<212> PRT
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Ala Ala Pro Pro Ile Gln Ser Arg Ile Val Gly Gly Trp Glu Cys Glu
            20                  25                  30
Gln His Ser Gln Pro Trp Gln Ala Ala Leu Val Met Glu Asn Glu Leu
        35                  40                  45
Phe Cys Ser Gly Val Leu Val His Pro Gln Trp Val Leu Ser Ala Ala
    50                  55                  60
His Cys Phe Gln Asn Ser Tyr Thr Ile Gly Leu Gly Leu His Ser Leu
65                  70                  75                  80
Glu Ala Asp Gln Glu Pro Gly Ser Gln Met Val Glu Ala Ser Leu Ser
                85                  90                  95
Val Arg His Pro Glu Tyr Asn Arg Pro Leu Leu Ala Asn Asp Leu Met
            100                 105                 110
Xaa Ile Lys Leu Asp Glu Ser Val Ser Glu Ser Asp Asn Ile Arg Xaa
        115                 120                 125
Ile Ser Ile Xaa Ser Gln Cys Pro Thr Ala Gly Asn Phe Cys Leu Val
    130                 135                 140
Ser Gly Trp Gly Leu Leu Ala Asn Gly Arg Met Pro Thr Val Leu Gln
145                 150                 155                 160
Cys Val Asn Val Ser Val Val Ser Glu Glu Val Cys Ser Lys Leu Tyr
                165                 170                 175
Asp Pro Leu Tyr His Pro Ser Met Phe Cys Ala Gly Gly Gly Gln Asp
            180                 185                 190
Gln Lys Asp Ser Cys Asn Gly Asp Ser Gly Gly Pro Leu Ile Cys Asn
        195                 200                 205
Gly Tyr Leu Gln Gly Leu Val Ser Phe Gly Lys Ala Pro Cys Gly Gln
    210                 215                 220
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Val Gly Val Pro Gly Val Tyr Thr Asn Leu Cys Lys Phe Thr Glu Trp
225                 230                 235                 240
Ile Glu Lys Thr Val Gln Ala Ser
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<211> 254
<212> PRT
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<400> 7

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Met Ala Thr Ala Gly Asn Pro Trp Gly Trp Phe Leu Gly Tyr Leu Ile
 1               5                  10                  15
Leu Gly Val Ala Gly Ser Leu Val Ser Gly Ser Cys Ser Gln Ile Ile
            20                  25                  30
Asn Gly Glu Asp Cys Ser Pro His Ser Gln Pro Trp Gln Ala Ala Leu
        35                  40                  45
Val Met Glu Asn Glu Leu Phe Cys Ser Gly Val Leu Val His Pro Gln
    50                  55                  60
Trp Val Leu Ser Ala Ala His Cys Phe Gln Asn Ser Tyr Thr Ile Gly
65                  70                  75                  80
Leu Gly Leu His Ser Leu Glu Ala Asp Gln Glu Pro Gly Ser Gln Met
                85                  90                  95
Val Glu Ala Ser Leu Ser Val Arg His Pro Glu Tyr Asn Arg Pro Leu
            100                 105                 110
Leu Ala Asn Asp Leu Met Leu Ile Lys Leu Asp Glu Ser Val Ser Glu
        115                 120                 125
Ser Asp Thr Ile Arg Ser Ile Ser Ile Ala Ser Gln Cys Pro Thr Ala
    130                 135                 140
Gly Asn Ser Cys Leu Val Ser Gly Trp Gly Leu Leu Ala Asn Gly Arg
145                 150                 155                 160
Met Pro Thr Val Leu Gln Cys Val Asn Val Ser Val Val Ser Glu Glu
                165                 170                 175
Val Cys Ser Lys Leu Tyr Asp Pro Leu Tyr His Pro Ser Met Phe Cys
            180                 185                 190
Ala Gly Gly Gly His Asp Gln Lys Asp Ser Cys Asn Gly Asp Ser Gly
        195                 200                 205
Gly Pro Leu Ile Cys Asn Gly Tyr Leu Gln Gly Leu Val Ser Phe Gly
    210                 215                 220
Lys Ala Pro Cys Gly Gln Val Gly Val Pro Gly Val Tyr Thr Asn Leu
225                 230                 235                 240
Cys Lys Phe Thr Glu Trp Ile Glu Lys Thr Val Gln Ala Ser
                245                 250
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<400> 8

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 1               5                  10                  15
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Gln Ala Ala Leu Val Met Glu Asn Glu Leu Phe Cys Ser Gly Val Leu
            20                  25                  30
Val His Pro Gln Trp Val Leu Ser Ala Ala His Cys Phe Gln Asn Ser
        35                  40                  45
Tyr Thr Ile Gly Leu Gly Leu His Ser Leu Glu Ala Asp Gln Glu Pro
    50                  55                  60
Gly Ser Gln Met Val Glu Ala Ser Leu Ser Val Arg His Pro Glu Tyr
65                  70                  75                  80
Asn Arg Pro Leu Leu Ala Asn Asp Leu Met Leu Ile Lys Leu Asp Glu
                85                  90                  95
Ser Val Ser Glu Ser Asp Thr Ile Arg Ser Ile Ser Ile Ala Ser Gln
            100                 105                 110
Cys Pro Thr Ala Gly Asn Ser Cys Leu Val Ser Gly Trp Gly Leu Leu
        115                 120                 125
Ala Asn Gly Arg Met Pro Thr Val Leu Gln Cys Val Asn Val Ser Val
    130                 135                 140
Val Ser Glu Glu Val Cys Ser Lys Leu Tyr Asp Pro Leu Tyr His Pro
145                 150                 155                 160
Ser Met Phe Cys Ala Gly Gly Gly Gln Asp Gln Lys Asp Ser Cys Asn
                165                 170                 175
Gly Asp Ser Gly Gly Pro Leu Ile Cys Asn Gly Tyr Leu Gln Gly Leu
            180                 185                 190
Val Ser Phe Gly Lys Ala Pro Cys Gly Gln Val Gly Val Pro Gly Val
        195                 200                 205
Tyr Thr Asn Leu Cys Lys Phe Thr Glu Trp Ile Glu Lys Thr Val Gln
    210                 215                 220
Ala Ser
225
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specific CTL. The DNA sequence encoding the epitope of SEQ ID NO: 715 is provided in SEQ ID NO: 716.

To identify the class I restricting allele for the P501S-derived peptide of SEQ ID NO: 712 and 715 specific response, each of the HLA B and C alleles were cloned from the donor used in the *in vitro* priming experiment. Sequence analysis indicated that the relevant alleles were HLA-B8, HLA-B51, HLA-Cw01 and HLA-Cw07. Each of these alleles were subcloned into an expression vector and co-transfected together with the P501S gene into VA-13 cells. Transfected VA-13 cells were then tested for the ability to specifically stimulate the P501S-specific CTL in ELISPOT assays. VA-13 cells transfected with P501S and HLA-B51 were capable of stimulating the P501S-specific CTL to secrete gamma-IFN. VA-13 cells transfected with HLA-B51 alone or P501S + the other HLA-alleles were not capable of stimulating the P501S-specific CTL. These results demonstrate that the restricting allele for the P501S-specific response is the HLAB51 allele. Sequence analysis revealed that the subtype of the relevant restricting allele is HLA-B51011.

To determine if the P501S-specific CTL could recognize prostate tumor cells that express P501S, the P501S-positive lines LnCAP and CRL2422 (both expressing "moderate" amounts of P501S mRNA and protein), and PC-3 (expressing low amounts of P501S mRNA and protein), plus the P501S-negative cell line DU-145 were retrovirally transduced with the HLA-B51011 allele that was cloned from the donor used to generate the P501S-specific CTL. HLA-B51011- or EGFP-transduced and selected tumor cells were treated with gamma-interferon and androgen (to upregulate stimulatory functions and P501S, respectively) and used in gamma-interferon Elispot assays with the P501S-specific CTL clones 4E5 and 4E7. Untreated cells were used as a control.

Both 4E5 and 4E7 efficiently and specifically recognized LnCAP and CRL2422 cells that were transduced with the HLA-B51011 allele, but not the same cell lines transduced with EGFP. Additionally, both CTL clones specifically recognized PC-3 cells transduced with HLA-B51011, but not the P501S-negative tumor cell line DU-145. Treatment with gamma-interferon or androgen did not enhance the ability of CTL to recognize tumor cells. These results demonstrate that P501S-specific CTL,

generated by *in vitro* whole gene priming, specifically and efficiently recognize prostate tumor cell lines that express P501S.

A naturally processed CD4 epitope of P501S was identified as follows.

CD4 cells specific for P501S were prepared as described above. A series of 16 overlapping peptides were synthesized that spanned approximately 50% of the amino terminal portion of the P501S gene (amino acids 1- 325 of SEQ ID NO: 113). For priming, peptides were combined into pools of 4 peptides, pulsed at 4 μg/ml onto dendritic cells (DC) for 24 hours, with TNF-alpha. DC were then washed and mixed with negatively selected CD4+ T cells in 96 well U-bottom plates. Cultures were re-stimulated weekly on fresh DC loaded with peptide pools. Following a total of 4 stimulation cycles, cells were rested for an additional week and tested for specificity to APC pulsed with peptide pools using γ-IFN ELISA and proliferation assays. For these assays, adherent monocytes loaded with either the relevant peptide pool at 4ug/ml or an irrelevant peptide at μg/ml were used as APC. T cell lines that demonstrated either specific cytokine secretion or proliferation were then tested for recognition of individual peptides that were present in the pool. T cell lines could be identified from pools A and B that recognized individual peptides from these pools.